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Abstract. The effects of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 on rhizosphere colonization, cyanide production, and growth of velvetleaf and corn was examined. When formulated in alginate beads and inoculated onto velvetleaf and corn plants (10^9 CFU/plant), only *P. putida* ATH2-1RI/9 consistently reduced velvetleaf growth. Neither isolate inhibited corn growth. Interestingly the levels of *P. putida* ATH2-1RI/9 in the velvetleaf rhizosphere were 1000-fold higher (7×10^7 CFU/g root) than the *A. delafieldii* ATH2-2RS/1 populations. Cyanide (53–68 mM/g root) was recovered from the *P. putida* ATH2-1RI/9-inoculated velvetleaf plants. In contrast both *A. delafieldii* ATH2-2RS/1 and *P. putida* ATH2-1RI/9 colonized the corn rhizosphere to the same extent ($1–5 \times 10^7$ CFU/g root), producing 1 mM and 14 mM/g root respectively. These results suggest that bacterial formulation methods can influence the effectiveness of deleterious rhizobacteria in reducing weed growth.

The introduction of bacteria into soil for weed control requires that the organisms survive and multiply under field conditions. van Veen et al. [23] noted that for microbes to be successful in the biological control of pest plants, colonization of soil and plant roots needs to be sufficiently high for the intended purpose. Several releases of microorganisms into soils, including microbes for weed control, have been successful [7, 14]. Kennedy et al. [11] found the introduction of weed-deleterious rhizobacteria (WRB) into soil effectively reduced downy brome growth in the field. A variety of liquid and solid formulations have been devised to promote rhizobacterial persistence in inoculated soil [9]. The semolina-flour-based granular formation termed "pesta" has been used successfully in formulating WRB for green foxtail [8]. Cell encapsulation in sodium alginate has been used to protect cells from environmental stresses, promoting bacterial survival and releasing the microbe slowly into the soil during poly-

mer degradation [4, 5, 20, 21, 26]. Relatively few studies describing the formulation of WRB in alginate have been published and none have addressed the success of alginate-formulated WRB in weed suppression [13].

The current study involved the use of alginate-encapsulated formulations of two cyanogenic bacterial isolates with biological control potential for the weed velvetleaf (*Abutilon theophrasti*). *Pseudomonas putida* ATH2-RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 were recovered from velvetleaf roots and reduce the growth of liquid-inoculated velvetleaf plants, but not corn plants, grown in autoclaved soil [16]. Cyanogenesis is a phenotype associated with plant suppression and may contribute to the ability of certain WRB to suppress weeds [1, 2, 12]. Due to changes that occur during the growth of bacteria [25], both stationary growth phase (SGP) and logarithmic growth phase (LGP) cells were formulated. The establishment of rhizosphere bacterial populations and cyanide levels and the accompanying effects on plant growth were used as indicators of bacterial compatibility with the alginate formulation.

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Table 1. Velvetleaf and corn rhizosphere colonization of weed deleterious bacteria. Levels of both logarithmic and stationary phase RS (*Acidovorax delafieldii* ATH2-2RS/1) bacteria were significantly ($p < 0.05$) lower on velvetleaf, but not corn, roots as compared with RI (*Pseudomonas putida* ATH2-1RI/9) bacteria. $n = 10–12$ plants from three independent experiments

	Mean ± 1 SE CFU/g root			
	Velvetleaf roots		Corn roots	
Bacterial growth phase	RI	RS	RI	RS
Logarithmic	$9.9 \pm 2.6 \times 10^7$	$2.0 \pm 1.2 \times 10^4$	$3.8 \pm 1.2 \times 10^7$	$1.4 \pm 1.2 \times 10^8$
Stationary	$7.1 \pm 2.3 \times 10^7$	$1.7 \pm 0.5 \times 10^4$	$4.3 \pm 1.3 \times 10^7$	$5.5 \pm 3.5 \times 10^7$

Materials and Methods

Bacterial cultures and alginate encapsulation. Spontaneous rifampicin-resistant mutants of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 were used in this study. These mutants produce cyanide and reduce velvetleaf growth (as a liquid inoculum) to approximately the same extent as wild-type bacteria. To establish logarithmic growth phase and stationary growth phase cells, bacteria were cultured at 28°C with shaking in tryptic soy broth (TSB) for 10 or 17 h (LGP for *Acidovorax delafieldii* ATH2-2RS/1 and *Pseudomonas putida* ATH2-1RI/9 respectively) or 18 or 24 h (SGP for *Acidovorax delafieldii* ATH2-2RS/1 and *Pseudomonas putida* ATH2-1RI/9 respectively). Cells were collected by centrifugation, washed in sterile dilution buffer (SDB: 10 mM potassium phosphate, 0.1 M NaCl, pH 7.2), and resuspended in one twentieth the original culture volume of TSB. Cells were encapsulated in alginate according to van Elsas et al. [22]. The alginate mixture contained 1% sodium alginate, 3% skim milk, and 3% bentonite in sterile TSB. Control formulations lacked bacteria. All alginate mixtures were extruded through a 22 gauge needle into sterile 0.1 M CaCl$_2$, allowed to harden for 1 h, and washed in sterile TSB. After incubation overnight at 28°C, the alginate beads were strained to remove excess liquid and stored at 4°C. Beads were used to inoculate plants within 3 weeks of preparation. Bacterial levels in alginate beads were quantified by dissolving in SDB followed by serial dilutions with spread plate counts on half-strength TSA containing 100 μg rifampicin/ml.

Plant growth experiments and rhizosphere analysis. Field soil was collected and processed according to Owen and Zdor [16]. The soil had not received herbicide treatment the previous year. Corn and field-collected velvetleaf seed were surface-disinfested, pregerminated, and planted in polypropylene tubes (length 110 cm, width 2.5 cm; using 45 g of autoclaved soil per tube according to Owen and Zdor [16]). Each seed received 10^9 colony forming units (CFU) of *Pseudomonas putida* ATH2-1RI/9 or *Acidovorax delafieldii* ATH2-2RS/1 grown to log or stationary growth phases or the equivalent mass of control beads (range 4–50 mg for *Pseudomonas putida* ATH2-1RI/9 and 5–33 mg for *Acidovorax delafieldii* ATH2-2RS/1), the experiment being repeated three times (3 or 4 plants per experimental group for each experiment). This level of bacteria has been found to reduce velvetleaf growth as a liquid inoculum [16]. After 3 weeks of growth at 26°C plants were carefully removed from the tubes and length and weight measurements of both roots and shoots recorded. Rhizosphere levels of the bacterial inoculum and cyanide were quantified according to Owen and Zdor [16] by treating rhizosphere samples with HCl and trapping the released cyanide in NaOH. Cyanide quantities were determined colorimetrically using *N*-chlorosuccinimide and hydantoin-pyridine reagents. Data were analyzed using analysis of variance (ANOVA) at the 95% confidence level. Pearson's correlation and two-way ANOVA were used to determine positive or negative significant trends.

Results and Discussion

Depending on the growth phase and bacterial isolate used, levels of inoculum bacteria ranged from 1×10^7 to 7×10^8 CFU/milligram of alginate beads. Both log and stationary growth phase cultures gave similar levels of bacteria in the inoculum (data not shown). The highest bacterial levels in the inoculant were achieved with *Acidovorax delafieldii* ATH2-2RS/1. These levels of encapsulated inoculum cells are about 10–100 times greater than those reported for alginate-encapsulated *P. fluorescens* [22]. The higher inoculum levels achieved in this study may be attributed to the use of a nutrient-rich growth medium (TSB) or differences between strains in survival during the formulation procedure.

When comparing rhizosphere inoculant levels from LGP versus SGP cells, there were no statistically significant differences between the two growth phases (Table 1). In contrast, significant strain–plant differences were noted. Although *Pseudomonas putida* ATH2-1RI/9 colonized velvetleaf and corn roots to similar levels (approx. 10^7 CFU/g root), velvetleaf rhizosphere *Acidovorax delafieldii* ATH2-2RS/1 populations were 10^3 lower than the equivalent populations on corn roots. Interestingly the highest inoculant rhizosphere populations were found with RS on corn roots (Table 1).

The highest levels of rhizosphere HCN were found with *Pseudomonas putida* ATH2-1RI/9-inoculated velvetleaf, and ranged from approximately 53 to 68 mM HCN/g root (Table 2). No significant difference in HCN levels occurred between plants inoculated with SGP and LGP *Acidovorax delafieldii* ATH2-2RS/1 or *Pseudomonas putida* ATH2-1RI/9. However, HCN levels from *Pseudomonas putida* ATH2-1RI/9-colonized roots were consistently higher than HCN levels from *Acidovorax delafieldii* ATH2-2RS/1-inoculated roots for both plant species. The lowest levels of HCN (0.5–1 mM HCN/g

Table 2. Rhizosphere HCN (μM/g dry roots) recovered from velvetleaf and corn plants 21 days after inoculation with RI (*Pseudomonas putida* ATH2-1RI/9) and RS (*Acidovorax delafieldii* ATH2-2RS/1) bacteria. Cyanide levels were significantly ($p < 0.05$) lower on roots of both plant types for RS-inoculated plants compared with RI-inoculated plants. $n = 10–12$ plants from three independent experiments.

| | Mean ± 1 SE μM/g dry root | | | |
| | Velvetleaf | | Corn | |
Bacterial growth phase	RI	RS	RI	RS
Logarithmic	53,524 ± 11,403	1029 ± 1029	14,772 ± 4080	1009 ± 581
Stationary	68,022 ± 19,249	724 ± 724	11,378 ± 3707	502 ± 179
Control	6791 ± 2135	279 ± 279	1564 ± 700	379 ± 140

root) were recovered from *Acidovorax delafieldii* ATH2-2RS/1-inoculated roots. Moreover only 1 plant of the 11–12 LGP and SGP *Acidovorax delafieldii* ATH2-2RS/1-inoculated velvetleaf roots had detectable cyanide levels. Cyanide levels were significantly higher in *Pseudomonas putida* ATH2-1RI/9-colonized velvetleaf roots than in *Pseudomonas putida* ATH2-1RI/9-colonized corn roots. Previous studies suggest that the growth phase of bacteria is important to the production of secondary metabolites such as phenazine-1-carboxylic acid and cyanide that are important in plant–microbe interactions [6, 17, 18]. Vanderhove et al. [24] also reported the influence of growth phase on the survival of *P. fluorescens* in soil. However, in the current study cells formulated at different stages of growth did not differ significantly in their rhizosphere populations and effect on cyanide levels and plant growth. This lack of difference may suggest that the stage of growth of these two WRB prior to alginate formulation has a minimal effect on the influence of the bacteria in the rhizosphere. It also may be that the formulation process minimizes any differences between LGP and SGP cells relative to rhizosphere colonization and cyanide production that exist before formulation.

Velvetleaf shoot length and fresh weight for both LGP and SGP *Pseudomonas putida* ATH2-1RI/9-inoculated plants were significantly ($p < 0.05$) reduced relative to control plants (Fig. 1). Similar comparison for shoot dry weight showed some reduction, but the difference was not statistically significant. Reductions in shoot growth of *Pseudomonas putida* ATH2-1RI/9-inoculated velvetleaf plants ranged from 68% to 87% of control plant values. *Acidovorax delafieldii* ATH2-2RS/1-inoculated velvetleaf plants did not show a statistically significant reduction for any of the velvetleaf shoot growth parameters compared with controls, with velvetleaf shoot measurements ranging from 88% to 100% of controls.

Velvetleaf root dry weight for both LGP and SGP *Pseudomonas putida* ATH2-1RI/9-inoculated plants

were statistically significantly lower than controls (Fig. 2). Root dry weight of LGP and SGP *Pseudomonas putida* ATH2-1RI/9-inoculated plants was 61% and 56% respectively of the control value. Both LGP and SGP *Acidovorax delafieldii* ATH2-2RS/1-inoculated root lengths were significantly shorter than those of control plants, with mean root lengths being 88% of control values. *Acidovorax delafieldii* ATH2-2RS/1 inoculation did not reduce root dry weight relative to control plants. Most inoculated velvetleaf root systems appeared less branched compared with controls. Growth comparisons for inoculated corn plants (Figs. 1, 2), with the exception of shoot fresh weight of SGP *Pseudomonas putida* ATH2-1RI/9-inoculated plants, showed no statistically significant difference relative to controls.

The field use of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 as weed control agents will necessitate an appropriate mode of storage and transfer to the target plants. These current results indicate that the alginate encapsulation method of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 is possible. Up to 10^{11} cells per gram of alginate beads were recovered from samples kept at 4°C for as long as 3 weeks. To simplify inoculant storage, ideally the beads should be in a dried form. Moist beads were used in this study because subsequent recovery of bacterial cells from dried preparations was significantly reduced (data not shown). van Elsas et al. [22] found that air-drying encapsulated bacteria in beads resulted in a significant reduction of viable cells compared with non-dried beads. Further research in the area of enhanced productivity and stability of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 in dried alginate beads is needed. Hall and colleagues [10] tested the transport and survival of alginate-encapsulated *Pseudomonas aeruginosa* through soil microcosms. They reported the greatest survival rates and soil distribution when using alginate-encapsulated inoculants. Alginate bead breakdown should act to release cells into the soil matrix, resulting in increased
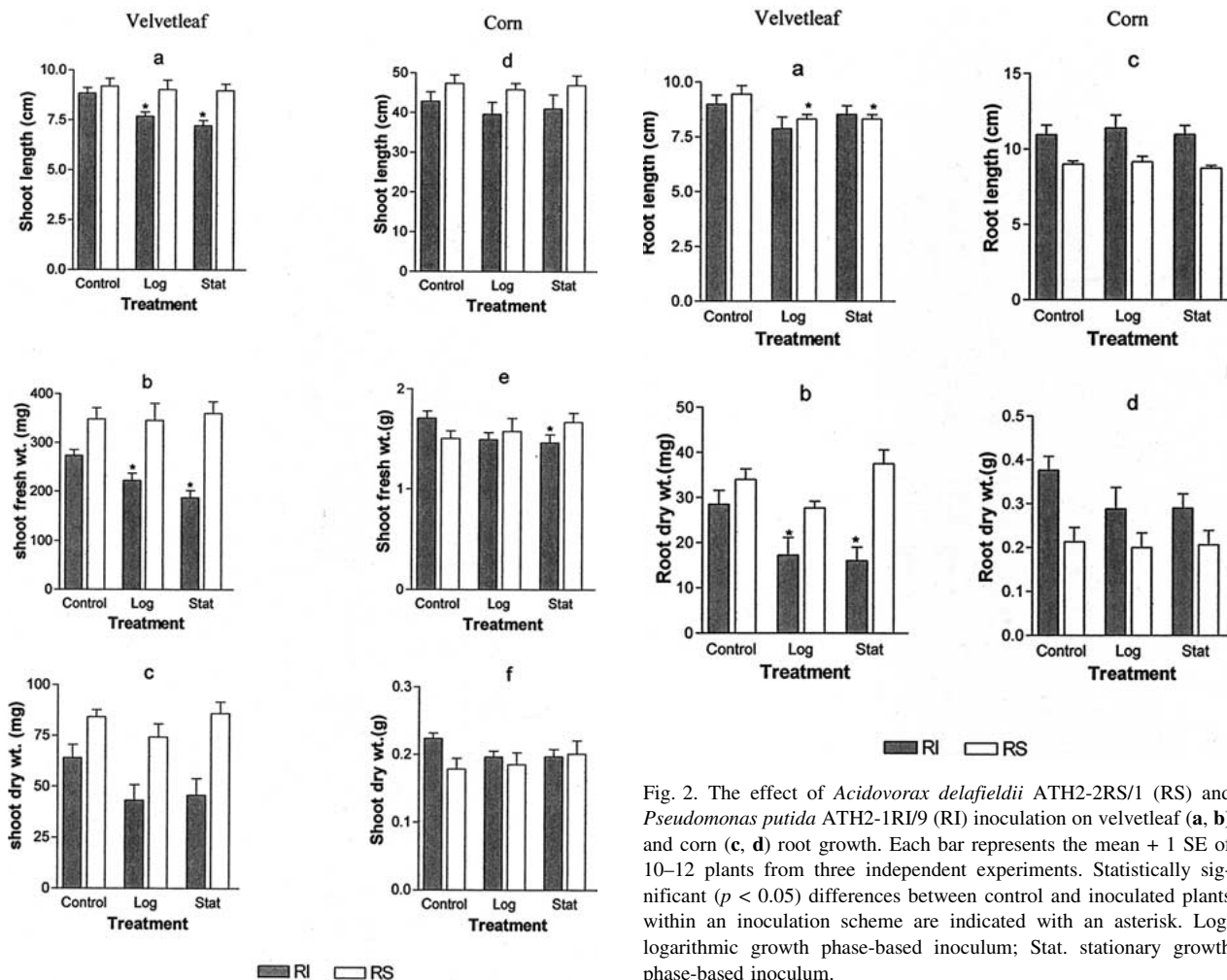
Fig. 1. The effect of *Acidovorax delafieldii* ATH2-2RS/1 (RS) and *Pseudomonas putida* ATH2-1RI/9 (RI) inoculation on velvetleaf (**a–c**) and corn (**d–f**) shoot growth. Each bar represents the mean + 1 SE of 10–12 plants from three independent experiments. Statistically significant ($p < 0.05$) differences between control and inoculated plants within an inoculation scheme are indicated with an asterisk. Log, logarithmic growth phase-based inoculum; Stat, stationary growth phase-based inoculum.

Fig. 2. The effect of *Acidovorax delafieldii* ATH2-2RS/1 (RS) and *Pseudomonas putida* ATH2-1RI/9 (RI) inoculation on velvetleaf (**a**, **b**) and corn (**c**, **d**) root growth. Each bar represents the mean + 1 SE of 10–12 plants from three independent experiments. Statistically significant ($p < 0.05$) differences between control and inoculated plants within an inoculation scheme are indicated with an asterisk. Log, logarithmic growth phase-based inoculum; Stat. stationary growth phase-based inoculum.

persistence of the encapsulated agent in the rhizosphere of a target plant. Smit et al. [19] demonstrated the protective effect of alginate encapsulation of *Pseudomonas fluorescens* against lysis by bacteriophage ΦR2f in soil. Considering that the initial inoculant levels were approximately 2.2×10^7 cells/g soil, significant levels (10^7 CFU/g root) of *Pseudomonas putida* ATH2-1RI/9 cells were recovered from the rhizosphere. However, there was a significant reduction in the number of *Acidovorax delafieldii* ATH2-2RS/1 cells recovered from velvetleaf, but not corn. In this current study, the failure of *Acidovorax delafieldii* ATH2-2RS/1 to establish

higher rhizosphere populations was reflected in minimal recovery of HCN from *Acidovorax delafieldii* ATH2-2RS/1-inoculated rhizospheres and the concomitant lack of effect on plant growth. This seems to indicate that increased levels of both bacteria and HCN correlate with reduction in some growth patterns and organ mass of the target plant. Previous work by Owen and Zdor [16] indicated that inoculation of velvetleaf and corn plants with liquid suspensions of *Pseudomonas putida* ATH2-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 resulted in significant reductions in certain growth parameters of velvetleaf but not corn. It was suggested that the negative effect on velvetleaf may be due to metabolites of the bacteria such as HCN that may affect root metabolism and development by inhibiting enzymes such as cytochrome oxidase [3].

The basis for why *Acidovorax delafieldii* ATH2-2RS/1 effectively colonized the corn rhizosphere, but not the velvetleaf, rhizosphere as an alginate bead

inoculum is unclear. It is possible that the release of bacterial cells from the beads in the rhizosphere differs between the two plants. This isolate did effectively colonize the velvetleaf rhizosphere as a liquid inoculum [16]. Presumably, there is some factor present or absent in the association with velvetleaf that differs in the association with corn roots. For example, the fibrous root system of the monocot may offer increased surface area, increasing the chance of contact for colonization when a liquid, but not a solid, inoculum is used. Such differential colonization based on variations in root system physiology is supported by the work of Nijhuis et al. [15], who found that grass rhizobacterial isolates from the grass rhizosphere exhibited distinct patterns of colonization at different locations of the same root system.

The current study suggests that alginate encapsulation is more compatible with *Pseudomonas putida* ATH2-1RI/9 than *Acidovorax delafieldii* ATH2-2RS/1. Thus, at least for the *Pseudomonas putida* ATH2-1RI/9 isolate, alginate formulation proved appropriate, allowing for stabilization, handling, and persistence of the organism at the target site. The differential success of the two alginate-formulated WRB isolates in the plant rhizosphere underlines the need to examine the response of specific WRB isolates to formulation in designing WRB-based weed control strategies.

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